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Award Number: W81XWH-07-2-0020

TITLE: Project 3 - Molecular Evolution of Human PON to Design Enhanced Catalytic Efficiency for Hydrolysis of Nerve Agents

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REPORT DATE: February 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
01-02-2009

2. REPORT TYPE
Annual

3. DATES COVERED
29 JAN 2008 - 28 JAN 2009

4. TITLE AND SUBTITLE

Project 3 - Molecular Evolution of Human PON to Design Enhanced Catalytic Efficiency for Hydrolysis of Nerve Agents

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-2-0020

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Weizmann Institute of Science
Rehovot 76100, Israel

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The long-term objective of this effort is to develop a generic gene shuffling-based technology to rapidly screen libraries of 1010 proteins/peptides encoded by DNA libraries, for identifying biomolecules that can intercept both existing and emerging organophosphate-based chemical warfare nerve agents (CWNA). The specific milestones for year 1 were (a) Proof of concept for the proposed technology; (b) Generation of 4-6 libraries based on recombinant PON1 and PON3 based on gene shuffling and random mutations (c) synthesis of OP model compounds. In general, all 1st year milestones were met and well beyond, specifically (i) the generation of recombinant PON3 variants and (ii) generation of recombinant PON1 variants, two of them (3B3 and 2B4) were overexpressed, purified and found to have a 72- and 13-fold increased kcat/Km relative to the wild-type like enzyme toward a cyclosarin model compound. Notably the increased activity of these variants seems to be highly selective, e.g. the activity of the two variants with the diethyl phosphoryl analog DEPCyC shows one to be lower and the other to be only 3-fold higher than the wild-type PON1. Relevance: this technology is envisaged to provide rapid discovery of pretreatment and post challenge therapeutic drugs against existing and emerging CWNA threats and will shorten the time from emergence of a threat to identification of potential counter-measures to a few days or weeks.

15. SUBJECT TERMS
Directed evolution, X-ray crystallography, organophosphates & bioscavangers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	18	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

I. Specific Aims

1. The development of high-throughput assays for OP hydrolase variants exhibiting high specificity and turnover.
2. Provision of proof-of-concept for the proposed core technology employing directed evolution of new recombinant PON and AChE variants.
3. Isolation of interceptors for G- and V-type nerve agents, and their expression in soluble form.
4. Design, generation and selection of 2nd generation libraries for V- and G-type agents.
5. Large-scale production of selected enzyme candidates, and their kinetic, structural and pharmacological evaluation.
6. Establishment of “off-the-shelf” libraries for rapid identification of antidotes against emerging threats

II. Significance to the goals of CounterACT

The proposed approach opens up new opportunities for rapid identification, characterization and implementation of novel countermeasures against CW agents. It will significantly decrease the time interval between the appearance of a new threat and the discovery of potential antidotes to counteract it. The major benefits will be one or more products capable of efficient catalytic hydrolysis of G- and V-type nerve agents, as well as gene libraries derived from existing enzymes that can be used “off-the-shelf” to isolate new protein variants for almost any nerve agent or toxic industrial chemical serving as a target for the screen.

In general, all 3rd year milestones have been met, and additional tasks were performed to advance the goals of the project.

III. **03 Milestone #1**: **Develop at least two promising PON1 candidates capable of hydrolyzing the P(-) isomers of methylphosphonyl coumarin analogs of nerve agents**

In the 3rd year, special emphasis was placed on reversal of the stereo-selectivity of rePON1, so as to yield variants capable of hydrolyzing the toxic isomers of nerve agents with high values of k_{cat}/K_m. Milestone #1 success criteria were:

a) PON1 mutants should display k_{cat}/K_m values $>10^5$ $M^{-1}min^{-1}$ towards (-)-OPs (*viz.,* >2,000-fold greater than the wt enzyme), and we also proposed to:
b) Obtain at least one additional PON1 candidate with k_{cat}/K_m > $2x10^7$ $M^{-1}min^{-1}$ when acting upon racemic methylphosphonyl coumarin analogs

Our screening for reversed stereo-selectivity of PON1 variants towards chiral OPs utilized the (-) optical isomer of $CH_3P(O)(OiPr)$–X (IMP), and $CH_3P(O)$(O-cyclohexyl)–X (CMP), where the leaving group, X, is 3-cyano-7-hydroxy-4-methylcoumarin. (-)-IMP and (-)-CMP were obtained by treatment of the corresponding racemic OP ligands with the 3B3 rePON1 mutant, which hydrolyzes almost exclusively the (+)- isomers. In addition, (-)-CMP was isolated (on a 30-50 mg scale) by a chemo-enzymic procedure in which the non-hydrolyzed (-)-CMP residue from incubation of a 120 mg racemic mixture of CMP with the 3B3 mutant was extracted into chloroform, and then washed with a bicarbonate solution to remove excess of the free leaving group, 3-cyano-7-hydroxy-4-methylcoumarin. The (-)-CMP obtained after evaporation of the organic solvent was then crystallized; X-ray analysis confirmed the P_S absolute configuration of the toxic (-)-CMP isomer (Fig 1). The relatively small background fluorescence due to the presence of <1.5 % of free coumarin, and the absence of the (+) isomer, permitted meaningful and efficient FACS sorting of clones of variants capable of reversing the stereo-preference of rePON1 (Fig. 2), as well as unambiguous determination of their K_m and k_{cat} values for the toxic isomer. The new FACS-screened protocol based on the crystallized (-)-CMP expedited selection of active variants from 10^6-size libraries.

The enhanced evolution pathways for isolation of clones capable not only of reversing the stereo-preference from the (+) to the (-) isomer, but also of displaying high catalytic proficiency towards the toxic isomer, employed several engineering methodologies. We started from designed mutations based on the wt G3C9 variant. Thus, the double-mutant, H115W/V346A, was used to construct a 1st generation library containing mutants with up to 7-fold improved activity relative to H115W/V346A. Screening with (-)-IMP revealed that the F222S mutation is present in most of the improved clones. In a second round of mutagenesis, selecting with (-)-IMP, V346A disappeared from active clones, while H115W and F222S were retained. The next round of mutagenesis, to produce a 3rd generation library, involved screening with both (-)-IMP and (-)-CMP, thus permitting selection of mutants with preferential action on one or the other of these two model compounds. Thus, 3A7 was ~9 fold more active on (-)-IMP compared to (-)-CMP, while 8C8 displayed ~ 4-fold enhanced efficiency towards (-)-CMP relative to (-)-IMP. The latter mutant displayed a remarkable reversed stereo-selectivity towards the toxic (-)-CMP, with a k_{cat}/K_m of ~ 10^5 $M^{-1}min^{-1}$ (Appendices 3 & 4). Yet it should be noted that 8C8 retains its ability to hydrolyze the (+) isomer, albeit at a significantly slower rate relative to (-)-CMP.

Since a 4th generation library, generated and screened similarly, did not reveal significant improvement over 8C8, we designed substitution-saturation libraries based on the observation that mutations at H115 and H134 (the His dyad) seem to display an allosteric effect that displaces the catalytic Ca^{2+} away from its coordinating residues (see milestone #3 below). Saturation mutagenesis at selected positions utilized the rePON1 gene bearing the H115W mutation, and active mutants were selected from this library using FACS (by means of the double-emulsion technique) and racemic CMP. Positive clones were grown in 96-well plates, followed by activity measurements using the (-)-CMP isomer. The following four key mutations were found to be associated with enhanced activity towards the (-) isomer: L69G/A, H115W, H134R, and F222S. These residues, that control stereo-selectivity towards OP substrates, appear to be on one side of the catalytic cavity of rePON1. These mutants were shuffled by random mutagenesis at a low rate, and sorted by FACS. Some of the most active variants were purified and analyzed. The results are summarized in Table 1 (Appendix 4).

Table 1 shows that in most of the improved variants, we found five key mutations, *viz.,* L69G, H115W, H134R, F222S and T332S. However, the latter position is facing H115W on the opposite face of the cavity. One variant, 3D8, has an additional mutation, M196V, on the same side as the 4 mutations, L69G/A, H115W, H134R, and F222S. 3D8 is, thus far, the most active variant, exhibiting a k_{cat}/K_m value of $>10^7$ $M^{-1}min^{-1}$,*viz.*, $>4x10^5$-fold greater than the wt G3C9 variant, which displays extremely low activity on (-)-CMP. The K_m value of 3D8 (25 μM) indicates high affinity for the enzyme, making it a most suitable candidate for *in vivo* detoxification. In addition, 3D8 is ~50 fold more selective for (-)-CMP than for its non-toxic enantiomer, (+)-CMP, a difference that might be utilized to isolate large quantities of the (+)-CMP isomer by the chemo-enzymic protocol described above for purification of (-)-CMP. Overall, the 3D8 variant had the broadest specificity for the *O*-cyclohexyl, *O*-isopropyl and *O*-ethyl toxic isomers of methylphosphonyl coumarin, i.e. the (-) enantiomers, with the following k_{cat}/K_m values: $11.6x10^6$, $4.6x10^6$, and $6.2x10^6$ $M^{-1}min^{-1}$, respectively.

We thus demonstrated the potential of an enhanced evolution strategy, combining random and designed mutations, to yield a mutant with catalytic proficiency approaching the value that qualifies a catalytic bioscavenger as a candidate drug for pre-treatment of OP intoxication ($k_{cat}/K_m \sim 5x10^7$ $M^{-1}min^{-1}$). The next step was to examine the suitability of the coumarin derivatives used to select active mutants (*i.e.,* IMP and CMP; see structures in Table 2) as model OP substrates for identification and selection of clones relevant to the primary mission of this project, namely, to evolve a rePON1 capable of rapidly hydrolyzing the corresponding G-agents. To this end, we generated, *in situ*, in dilute aqueous solutions, the fluoridate analogs of IMP and CMP in which the P-O-coumarin moiety had been replaced by a P-F bond. The procedure implemented is fully controlled, safe and non-hazardous. Following complete release of the expected amount of the coumarin leaving group by NaF, in aqueous solution, the concentrations of the toxic (-)-IMP-F and (-)-CMP-F isomers were determined by titration of a known concentration of *Torpedo californica* AChE (*Tc*AChE), and were verified by measuring the bimolecular rate constants for inhibition of AChE by the *in-situ*-generated sarin and cyclosarin, which were found to be consistent with published data. k_{cat}/K_m values were determined by monitoring the loss of anti-*Tc*AChE potency of the freshly generated fluoridates, assuming that K_m is well above the concentration of the tested (-)-IMP-F and (-)-CMP-F which were held at 30-50 nM. Table 2 (Appendix #5) shows that mutants 0C9, 2D8, and 1A4 are 3-4-fold more potent towards (-)-cyclosarin than the corresponding coumarin analog, and all three variants catalyzed its hydrolysis with values of k_{cat}/K_m $>1x10^7$ $M^{-1}min^{-1}$. They were found to be only 3-to 4-fold less potent in catalyzing the IMP-fluoridate (sarin) when compared to IMP-coumarin. Thus, the observed broad specificity and high proficiency confirmed our assumption that the OP-O-coumarin substrates serve as reasonable predictors in the screening of PON1 variants for the capacity to detoxify G-type nerve agents. In view of the inability of human PON1 to hydrolyze *O*-pinacolyl methylphosphonyl-*O*-coumarin (PinMP) (*i.e.,* the soman analog model compound; Blum et al. [2008] *Biochemistry* **47**:5216-5224), it will be important to test the mutants listed in Table 2 on both PinMP-O-coumarin and on soman itself. Results shown in Table 2 should be verified using aqueous solutions of neat nerve agents at USAMRICD, Aberdeen, MD. Our data call for development of a protocol for screening existing and future libraries with the *in-situ*-generated fluoridates, which will permit direct sorting of mutants capable of efficiently hydrolyzing G-agents.

Finally, neutral drift libraries (Gupta and Tawfik [2008] *Nat Methods* **11**:939-42) selected with racemic CMP yielded a variant, 1G3, bearing the following mutations, T126A, A187V, F222S, H251N, Y293S, T332S. 1G3, like 3B3, catalyzed the hydrolysis of (+)-CMP with $k_{cat}/K_m = 1.8x10^7$ $M^{-1}min^{-1}$, *viz.,* 70-fold faster than the wt G3C9. This mutant, which was mentioned in our year-2 report, was re-expressed, purified, its mutations confirmed, and following evaluation of its kinetic constants, delivered to USAMRICD (see below milestone #2).

03 Milestone #2: Transfer of a minimum of 3 promising candidates (in addition to 2B4 and 3B3) to USAMRICD for testing with G and V-type nerve agents

Plasmids of variants 8C8, 2H4, and 1G3 were delivered for further evaluation against 'live' nerve agents at USAMRICD. In addition, the following rePON1 mutants are being expressed and purified in our laboratory, and will be transferred to USAMRICD before the end of February 2009: 2C3, 3D8, 8C8, 1G3, 3B3, 2H4, 0C9, 2D8 and 1A4.

03 Milestone #3: Provide detailed 3D structures of the H115W mutant and of the H115Q/H134Q double mutant of recombinant PON1

H115W, which displays enhanced hydrolysis of compounds containing the P-S bond, such as found in the nerve agent VX, and the H115Q/H134Q double-mutant, that is almost devoid of lactonase and aryl esterase activity (both mutants originate from the G2E6 variant), were over-expressed and crystallized, followed by determination of their crystal structures by X-ray crystallography. Examination of their 3D structures revealed the following:

a. *Movements of amino-acid side-chains and of the catalytic Ca^{+2} in the crystal structures of H115W and H115Q-H134Q relative to the wt PON1*.
   1. Movement of the catalytic Ca^{+2} by 1.7 Å (Appendix 6A)
   2. No changes observed in the side-chains coordinating to the structural Ca^{+2} (Appendix 6B)
   3. Whereas N224, N168 and N270 interact with the catalytic Ca^{+2} in the wt, they do not interact in either mutant (Appendix 7)
   4. Changes are seen in both mutants in the orientations of residues N224 and E53

b. *The Histidine dyad: H115, H134*
   1. Two alternative conformations (each at ~50% occupancy) were observed for H115 in the wt structure for which X-ray data were collected at pH 6.5, suggesting that the imidazole ring is mobile, and that there is ample free space to accommodation the corresponding conformers (Appendix #8A)
   2. The movement of the side-chains of mutated residues at position 115 is approximately towards the position of the alternative conformation of H115 in the observed structure of the wt enzyme collected at pH 6.5 (Appendix #8B).

The 3D structures suggest that the mutations at H115 and H134 have an allosteric effect that results in displacement of the catalytic Ca^{2+} from its coordinating residues in the wt enzyme, *viz.,* E53, D269, N168, N224 and N270. The movement of the catalytic Ca^{2+}, together with changes in the size and polarity of sub-sites in the active mutants identified (Table 1) may be correlated with both the enhancement of OPH activity and the reversal of stereo-selectivity. This suggests that construction of PON1 libraries based on the amino acid residues mentioned above, that coordinate to the catalytic Ca^{2+} ion in wt rePON, may produce improved PON1 mutants, and permit dissection of the structural features influencing binding and catalysis. Also, the presence of the phosphate ion, and its coordination to the catalytic Ca^{2+} in wt PON1, suggests that the catalytic Ca^{2+} may serve as an oxyanionic site for the tetrahedral OP substrate.

Additional progress in year 3:

(a) We have demonstrated by *in vitro* and *in vivo* studies (in collaboration with BioLine RX, Israel), both the improved thermal stability (ΔT_m of 13^{o}C in favor of rePON1 over-expressed in *E coli*) and the antidotal efficacy of rePON1, compared to PON1 purified from human serum, when tested against intoxication of mice with chlorpyrifos-oxon.

(b) In order to provide an independent source for the (+) and (-) isomers, we developed a stereo-specific protocol for the synthesis of the two enantiomers of $CH_3P(O)(OC_2H_5)$-O-coumarin (EMP). The (+) and (-) isomers were crystallized, and their absolute configurations were established by X-ray crystallography (Appendix #9).

IV. Year 03 Deliverables

(1) Mutants capable of hydrolyzing both isomers of CMP-coumarin and CMP-fluoride with values of k_{cat}/K_m >1x10^7 $M^{-1}min^{-1}$.
(2) Libraries of saturation-substitution mutants of rePON1, and a FACS screening protocol.
(3) 3D structures of wt rePON1 and of the two variants, H115W and H115Q/H134Q.
(4) Protocol for a controlled, safe and non-hazardous procedure for conversion of CMP and IMP bearing the coumarin leaving group to the corresponding fluoridates.
(5) Protocols for (a) stereo-specific synthesis of a chiral *O*-ethyl methylphosphonate with a 3-cyano-7-hydroxy-4-methylcoumarin leaving group (EMP); (b) Chemo-enzymic isolation of large quantities of a highly purified (-)-CMP derivative with a 3-cyano-7-hydroxy-4-methylcoumarin leaving group.

V. Year 03 Publications

Devi-Gupta, R. & Tawfik, D.S. (2008) Directed enzyme evolution via small and effective neutral drift libraries. *Nature methods* **11**: 2311-2318. (attached)

Tokuriki, N. & Tawfik, D.S. (2008) GroEL/ES chaperonins promote genetic variation and accelerate enzyme evolution. *Nature* (under revision)

Ashani, Y., Ben-David, M., Devi-Gupta, R., Greenblatt, H.M., Leader, H., Mullokandov, G., Silman, I., Tawfik, D.S., and Sussman, J.L. (2008) , Biochemical and Structural Analyses of Chiral Methylphosphonate Analogues of G- and V-agents for High-Throughput Screening of Reversed Stereoselectivity of Paraoxonase-1 Variants, Presentation at the 16^{th} Biennial Medical Defense Bioscience Review, USAMRICD, June 1-6, Marriott Hotel, Hunt Valley MD

Colletier, J.-P. et al Sussman, J.L., Silman, I. & Weik, M. (2008). Shoot-and-Trap: Use of specific x-ray damage to study structural protein dynamics by temperature-controlled cryo-crystallography. *PNAS* 105, 11742-7. (attached)

Xu, Y. et al Silman, I. & Sussman, J.L. (2008). Flexibility of Aromatic Residues in the Active-Site Gorge of Acetylcholinesterase: X-ray versus Molecular Dynamics. *Biophys J* 95, 2500-11. (attached)

Xu, Y. et al Silman, I., Sussman, J.L. & Weik, M. (2008). Induced-fit or pre-existing equilibrium dynamics? Lessons from protein crystallography and MD simulations on acetylcholinesterase. *Protein Sci* 17, 601-5. (attached)

VI. Year 03 Collaborations

1. Collaboration with Dr Haim Leader, a consultant in the Department of Materials and Interfaces at the Weizmann Institute of Science, on the stereo-specific synthesis of (+) and (-) EMP, and chemo-enzymic isolation of (-)-CMP.
2. Collaboration with Drs Jun Juang and John Cashman (HBRI, San Diego, CA) in the evaluation of 4 optical pairs of OP model compounds, bearing $S\text{-}CH_2CH_2N(CH_3)_2$ and $S\text{-}CH_2CH_2N^+(CH_3)_3$ leaving groups, as substrates for PON variants and as AChE inhibitors.
3. Collaboration with Drs Tom Magliery and Chris Hadad (Ohio State University, Columbus, OH) in attempting to obtain 3D structures of OP/rePON1 complexes by X-ray crystallography.
4. Collaboration with Drs Dave Lenz and Doug Cerasoli (USAMRICD, APG, MD) in testing PON1 variants provided by our laboratory on live agents.
5. Collaboration with BioLine RX,(Israel), in testing the *in vivo* efficacy of rePON1 as a treatment for OP intoxication

VII. Proposed Year 04 Milestones

During year 3 we developed the ‘neutral drift’ library concept, and designed mutations that led, via the techniques of directed evolution, to the production of several libraries from which we sorted mutants displaying activity on the toxic isomers of OP model compounds, CMP and IMP, that were found also to react rapidly with threat G-type nerve agents, with k_{cat}/K_m values approaching the required value of $5x10^7\ M^{-1}min^{-1}$. In view of the accumulated results, and of our success in demonstrating the ability of variants selected using model compounds to rapidly hydrolyze and detoxify sarin and cyclosarin *in vitro*, we wish to focus our 4th year efforts on the following milestones:

1. Development of a high-throughput screen, based on recombinant AChE, to evolve new PON1 variants capable of degradation of P-F-containing OPs at *in vivo* toxic concentrations (1 μM).

2. Kinetic and structural characterization of additional promising candidates. The criterion for success will be achievement of values of $k_{cat}/k_m > 1x10^7\ M^{-1}min^{-1}$ for hydrolysis of G-agents.

3. Synthesis and kinetic evaluation of the *O*-pinacolyl OP model substrate (*i.e.,* the soman analog) to ascertain the broad specificity of the sorted mutants (subjected to the availability of pinacolyl alcohol)

3. Over-expression and provision of the 3D structures of the most active mutants with and without the presence of OP ligands.

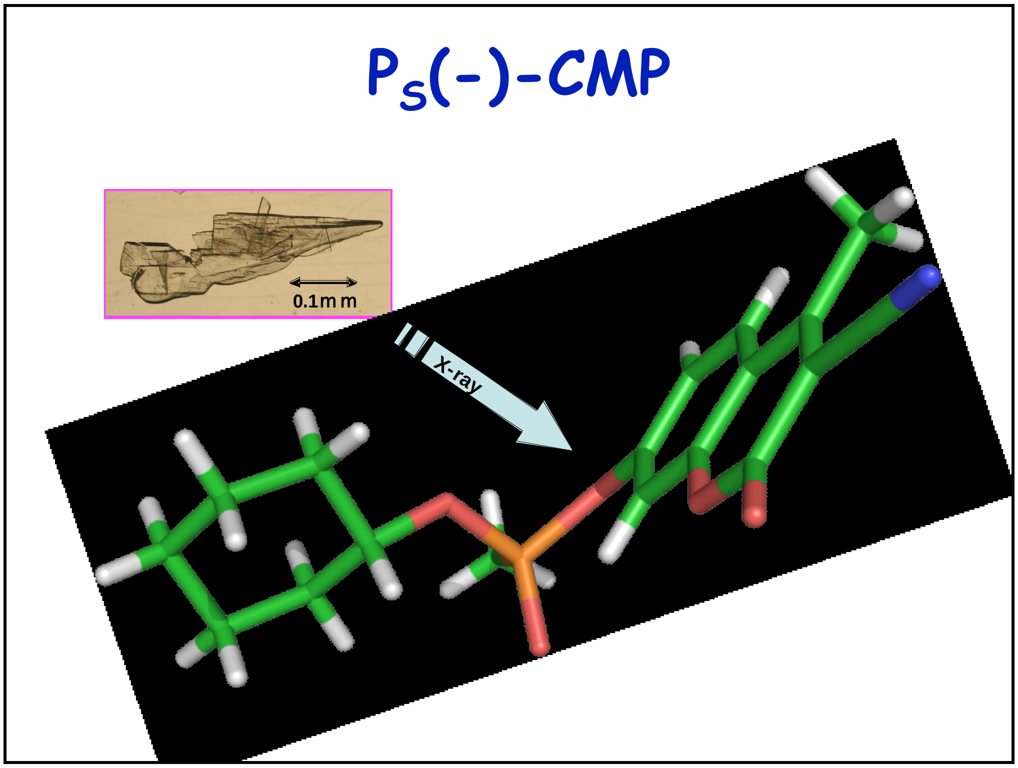
4. Transfer of the libraries and/or promising candidates to USAMRICD (ICD, Aberdeen MD) for *in vitro* and *in vivo* protection screens with nerve agents.

5. Generation and screening of PON1 libraries based on H115W for increased P-S/P-O hydrolytic activities (using parathiol and paraoxon), and for activity on a VX surrogate (amiton).

VIII. Proposed year 04 Deliverables

1. Protocol for a high-throughput screen, based on recombinant AChE, to screen directly for mutants hydrolyzing the corresponding OP fluoridates
2. Generation of 3rd generation libraries and their direct screening with P-F analogues at physiological concentration, with the aim of isolating mutants with a broader range of activities towards CMP, IMP and PinMP than the variants developed to date
3. Two new 3D structures of active rePON1 mutants

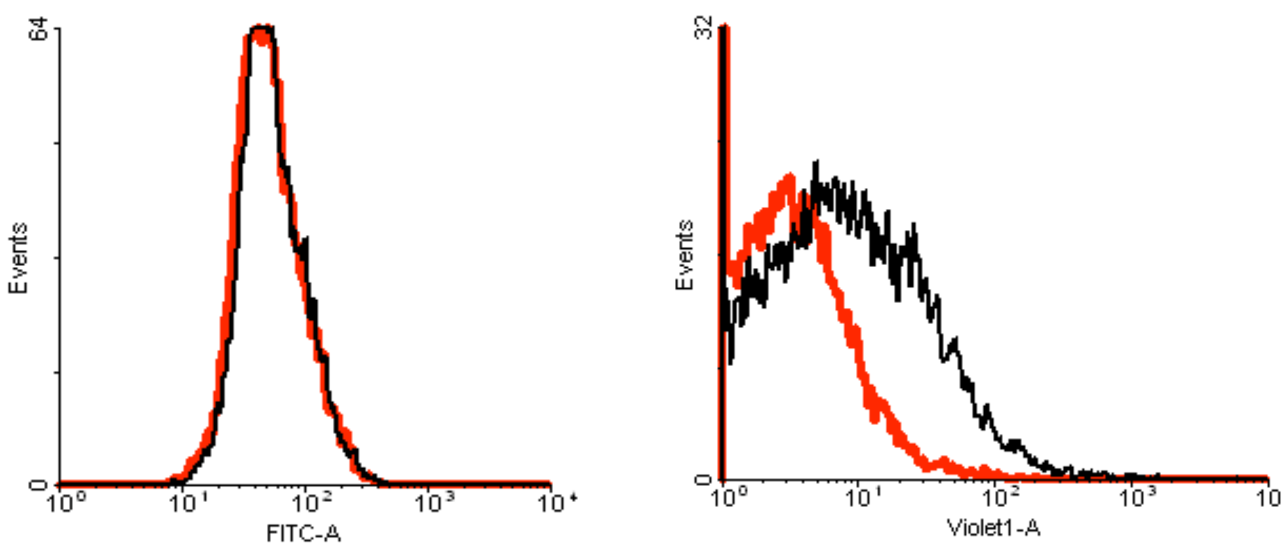
Appendix 1: **3D structure of (-)-CMP**



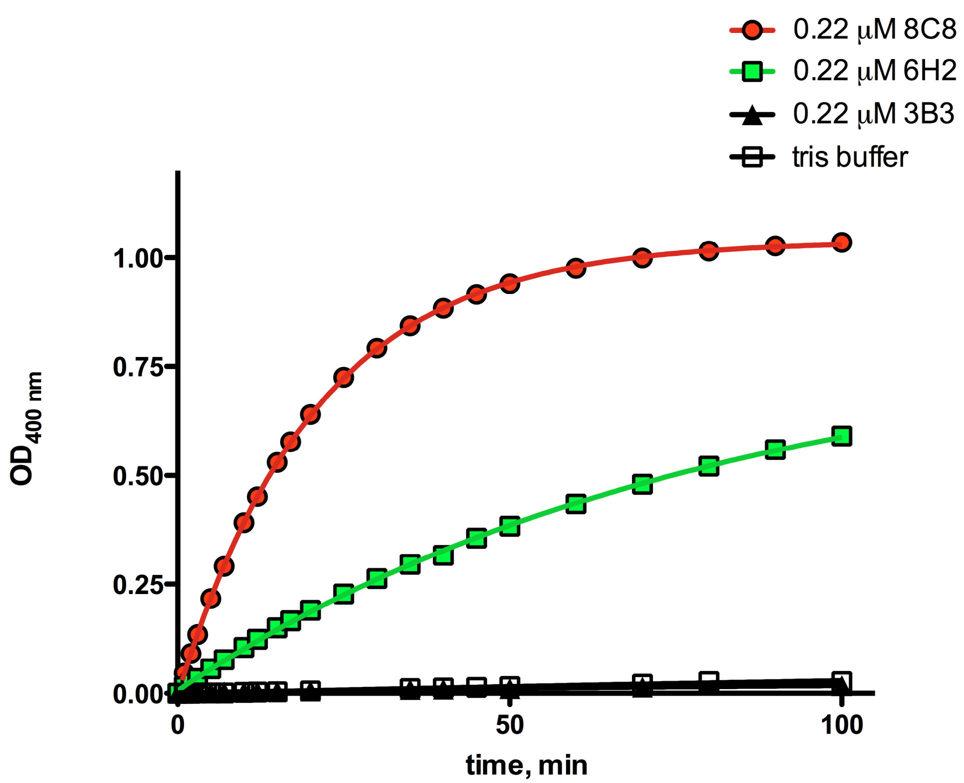
Appendix 2: **Sorting the double emulsion on FACS with (-)-CMP.**
Red - library; Black - positive controls

GFP

Product - coumarin leaving group



Appendix 3: **Reversed stereo-preference towards (-)-CMP displayed by mutants 8C8 and 6H2, as opposed to mutant 3B3, that essentially hydrolyzes only the (+)-CMP isomer**



Appendix 4: **Table 1. Catalytic efficiency of mutants selected from substitution library variants by (-)-CMP. Values shown are k_{cat}/K_m ($\mu M^{-1}min^{-1}$)**

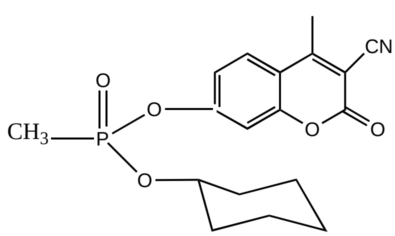
Variant	(-)-CMP	(-)/(+) ratio for CMP	(-)-IMP	Mutations
8C8	0.093±0.003 (1)[a]	27	0.022(1)[a]	L69S, V97A, H115W, P135A, F222S
2C3	0.7±0.01 (7.5)	8	0.079(3.6)	L69G, H115W, H134R, F222S, K233E
5H5	1.25±0.05 (13.4)	6	0.76(35)	L10S, F28Y, L69G, H115W, H134R, F222S, T332S
0C9	2.85±0.1 (31)	9	1.04(47)	L14M, L69G, S111T, H115W, H134R, F222S, T332S
2D8	3.52±0.13 (38)	7.5	0.85(39)	L69G, H115W, H134R, F222S, T332S
1A4	3.63±0.1 (39)	9.5	0.85(39)	A6E, L69G, H115W, H134R, F222S, K233E, T332S, T326S
3D8	11.6±0.23 (125)	47	4.6(209)	L69G, H115W, H134R, M196V, F222S, T332S

a. Figures in parentheses show enhancement relative to the 8C8 variant

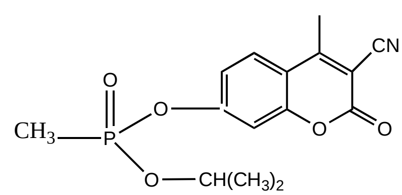
Appendix 5: Table 2: Comparison of catalytic activity on an OP-coumarin model compound of rePON1 variants selected from a saturation library by use of (-)-OP-coumarin compounds (see Table 1), and their OP-fluoridate analogs. The figures shown are values of k_{cat}/K_m x10^6 $M^{-1}min^{-1}$ analogs

mutant	(-)CMP-coumarin	(-)CMP-fluoridate	fluoridate/ coumarin	(-)IMP-coumarin	(-)IMP-fluoridate	fluoridate/ coumarin
8C8	0.09	0.2	2.2	0.02	0.03	1.5
3D8	11.6	3.3	0.3	0.46	0.10	0.2
0C9	2.8	11.1	3.9	1.04	0.32	0.3
2D8	3.5	14.3	4.1	0.85	0.23	0.3
1A4	3.6	11.3	3.1	0.85	0.21	0.25
2C3	0.7	0.47	1.5	0.08	0.02	0.25

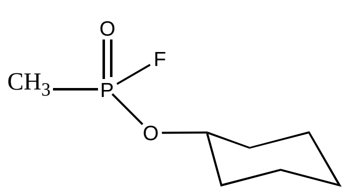
1. Data for OP-coumarin are based on release of the chromophore monitored at 400 nm.
2. The k_{cat}/K_m values for the fluoridates were determined by monitoring the rate of loss of anti-AChE potency of the *in situ*-generated compound, assuming K_m>>[P-F]. Calculations are based on a single enzyme concentration selected to allow convenient and reliable determination of the apparent k_{obs} of loss of anti-AChE potency.
3. The coumarin leaving group is displaced by fluoride from the racemic coumarin-containing OPs to yield CMP-fluoride and IMP-fluoride. It should be noted that for both types of OP the above data relate to the toxic (-) isomers



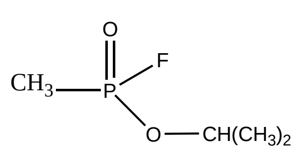
CMP-coumarin



IMP-coumarin



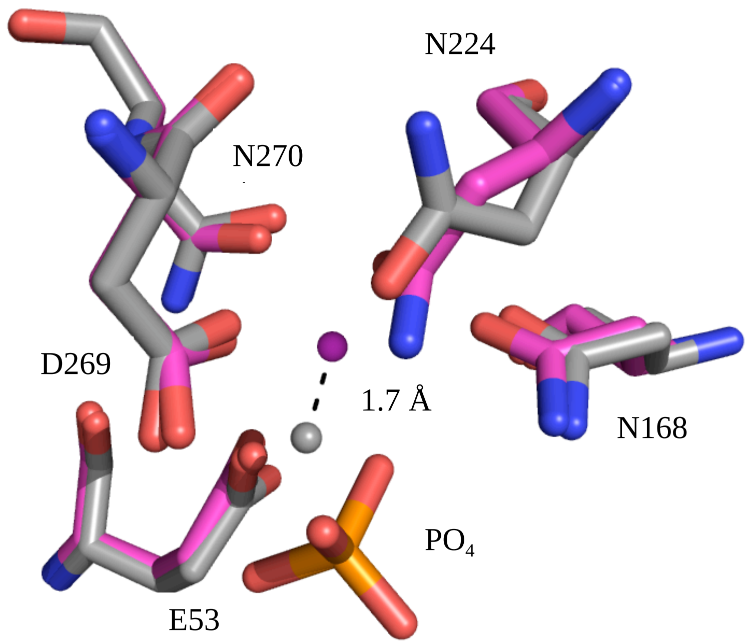
CMP-fluoride



IMP-fluoride

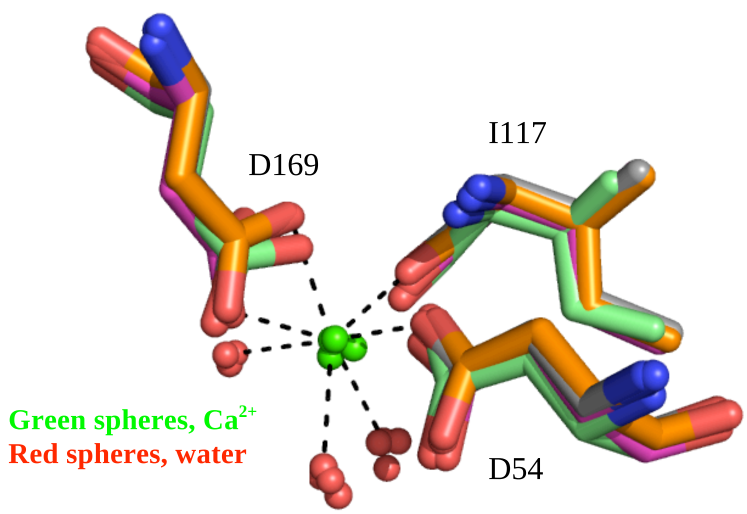
Appendix 6A: **Movement of the catalytic Ca^{+2} ion by 1.7 Å**.

Pink, wt rePON1, pH 4.6; Grey, H115W, pH 6.5



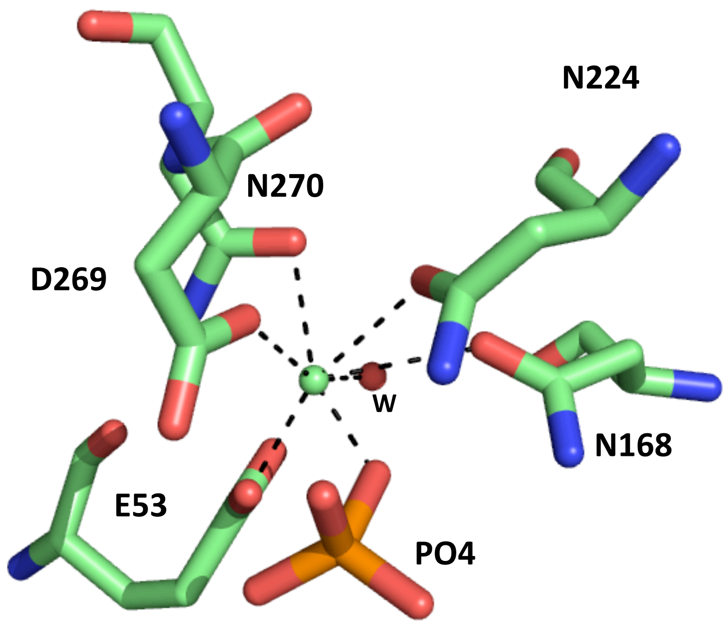
Appendix 6B: **Superposition of the structural Ca^{2+} ion of PON1 variants**

Pink, wt, pH 4.6; green, wt, pH, 6.5; grey, H115W, pH 6.5; orange, H115Q/H134Q, pH 6.5



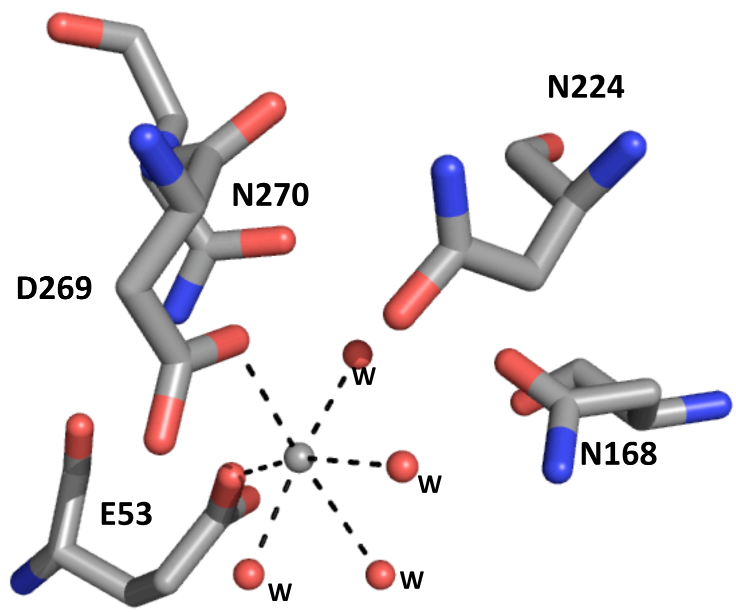
Appendix 7: **The catalytic Ca $^{2+}$ site of wt rePON1 (A) and H115W (B) (both at pH 6.5), showing the changes in coordination to the Ca $^{2+}$ ion.**

A



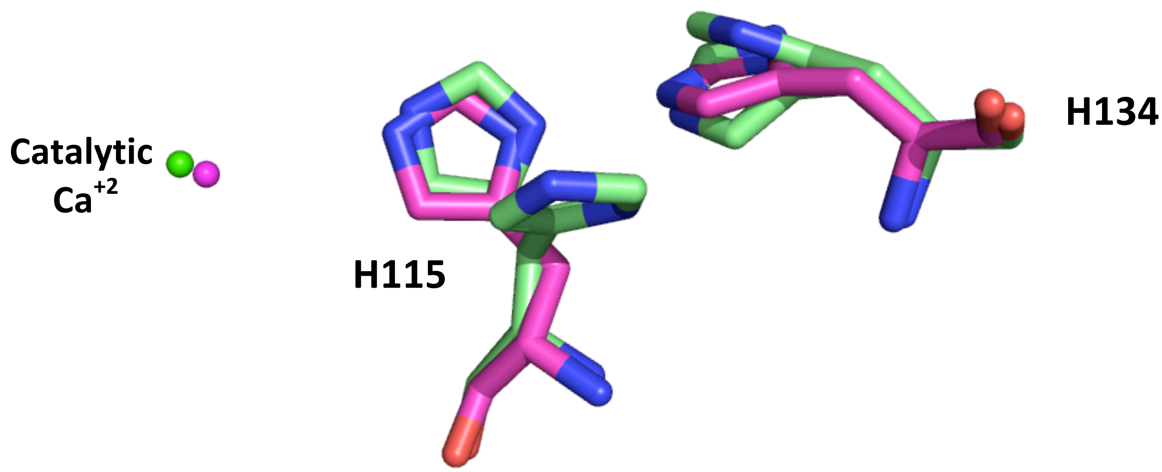
W=water molecule

B

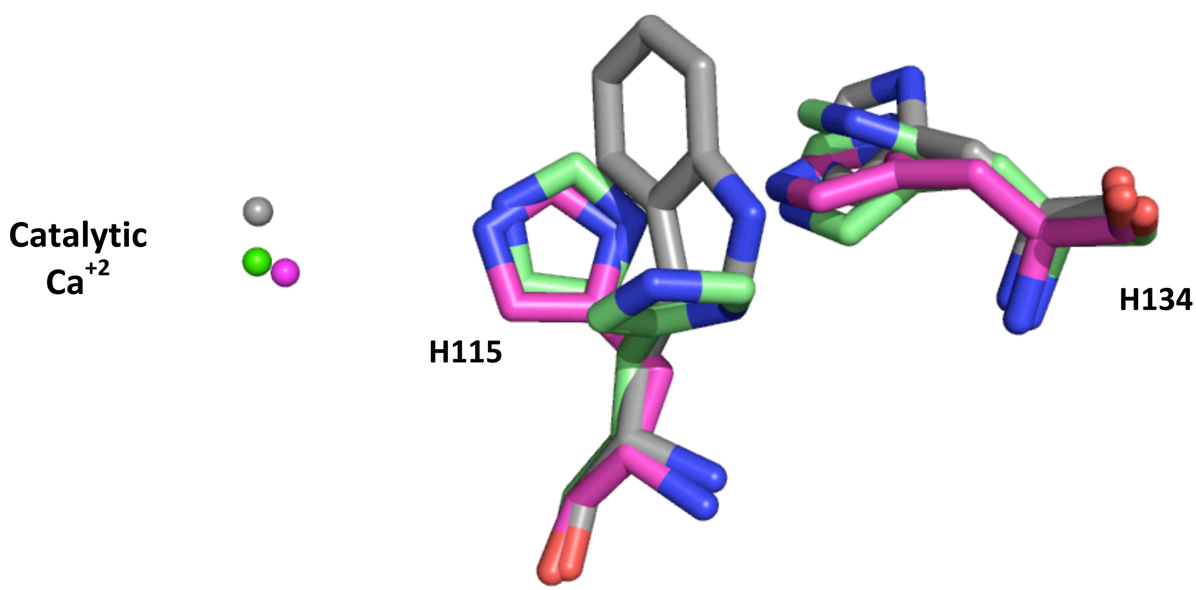


W=water molecule

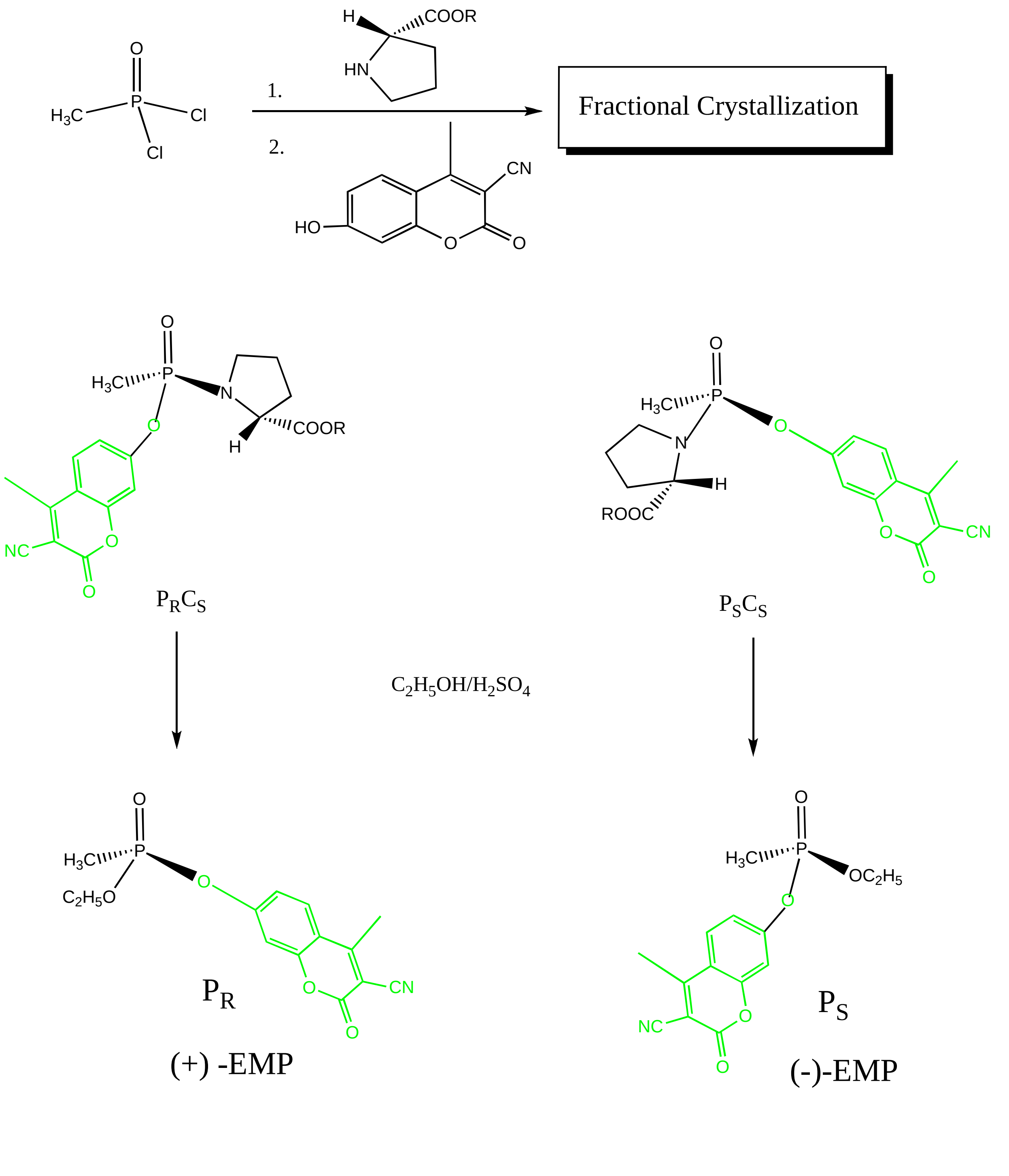
Appendix 8A: **Conformational mobility of the H115/H134 of wt rePON1 at pH 6.5 (green), compared to pH 4.6 (pink)**



Appendix 8B: **Superposition of mutant H115W (grey) and wt rePON1 at pH 4.5 (pink) and pH 6.5 (green)**



Appendix #9: **Stereo-specific synthesis of (+)- and (-)-EMP**



For New and Competing Applications (PHS 398) – DO NOT SUBMIT UNLESS REQUESTED
For Non-competing Progress Reports (PHS 2590) – Submit only Active Support for Key Personnel

PHS 398/2590 OTHER SUPPORT

Provide active support for all key personnel. **Other Support includes all financial resources, whether Federal, non-Federal, commercial or institutional, available in direct support of an individual's research endeavors, including but not limited to research grants, cooperative agreements, contracts, and/or institutional awards.** Training awards, prizes, or gifts do not need to be included.

There is no "form page" for other support. Information on other support should be provided in the *format* shown below, using continuation pages as necessary. ***Include the principal investigator's name at the top and number consecutively with the rest of the application.*** The sample below is intended to provide guidance regarding the type and extent of information requested.
For instructions and information pertaining to the use of and policy for other support, see Other Support in the PHS 398 Part III, Policies, Assurances, Definitions, and Other Information.
Note effort devoted to projects must now be measured using person months. Indicate calendar, academic, and/or summer months associated with each project.

Tawfik, D.S.
ACTIVE

Israel Science Foundation (ISF) Contract No. 274/05 Directed enzyme evolution (PI: Dan Tawfik) The goal is to apply directed enzyme evolutionusing gene libraries that carry insertion, deletions, duplication and fusion of entire gene segments, and circular permutations (rather than the traditional point mutation libraries).	1/10/2005 – 30/9/2009 1,356,000 IS	2.4 calendar
EU Sixth framework program Contract No. 028417 Microfluidic microdroplet femtoreactors (PI: Florian Hollfelder (University of Cambridge, UK, and 5 coPIs including Dan Tawfik) The goal of this consortium is the development of microfluidic devices and in vitro compartmentalization for high-throughput screening and directed evolution.	15.11.06-14.11.09 351,016 Euro	
EU Sixth framework program Contract No. 043340 Engineered Modular Bacterial Photoproduction of Hydrogen (PI: Ecole Polytechnique, Paris, and 5 coPIs including Dan Tawfik) The goal is to establish a systematic engineering methodology with the aim of generating a cyanobacterium that produces hydrogen.	15.1.07-14.1.10 291,598 Euro	
Ortho Clinical Diagnostics Contract No. 9827 Novel serum paraoxonase sera tests (PI: Michael Aviram & Dan Tawfik) The goal is the development of new sera tests for the stability and activity of the enzyme serum paraoxonase.	1.3.07-30.8.08 $69,700	0 calendar
DTRA Contract No. HDTRA 1-07-C-0024 Ultra-High-Throughput Screens for Rapid Identification of Potential Therapeutic Biomolecules for Existing and Emerging Threats (PIs: Dan Tawfik & Joel Sussman) The goal of this project is to develop high-throughput screening technologies for the rapid identification of biomolecules capable of intercepting chemical and biological war agents	1/6/2007 – 30/05/2009 Possible extension for another year $449,993 per annum	2.4 calendar

PENDING *(summarized for each individual)*		

Sussman, J.L ACTIVE		
EC Vth Framework Integrated Project Contract No. 031220 SPINE2-COMPLEXES From Receptor to Gene: Structure of Complexes (PI Stuart (Oxford Univ) with 19 other partners including CoPI Sussman) The major goal of this project is to study 3D structures of protein complexes involved in a number of signaling pathways involved in human health and disease….	7/1/2006 – 12/31/2010 250,000 Euro	1.2 calendar
EC Vth Framework Integrated Project Contract No. 037198 TEACH-SG – Training for High Volume, High Value Structural Genomics Methodologies (PI Stuart (Oxford Univ) with 19 other partners including CoPI Sussman) TEACH-SG aims to build on the successes of SPINE (Structural Proteomics IN Europe; FP5 Integrated Project) and other FP6 structural genomics projects to provide a platform for training young scientists and those from smaller laboratories and new EU member states in the state of the art technologies developed in Structural Genomics, particularly in high throughput techniques.	1/1/2007 – 6/30/2010 100,000 Euro	0.5 calendar
EC VIIth Framework Infrastructure Grant (Combination of CP & CSA) Contract No. 211252 INSTRUCT – Integrated Structural Biology Infrastructure (PI Stuart (Oxford Univ) with 5 other partners including CoPI Sussman) INSTRUCT aims to set up a framework consisting of distributed centres, each of which will maintain a set of core technologies such as protein production, NMR, crystallography and different forms of microscopy, including electron microscopy and combine this with a specific biological focus that will drive the development of technological and methodological expertise, notably for the analysis of functional complexes.	4/1/2008 – 3/31/2010 235599 Euro	0.5 calendar
DTRA Contract No. HDTRA 1-07-C-0024 Ultra-High-Throughput Screens for Rapid Identification of Potential Therapeutic Biomolecules for Existing and Emerging Threats (PIs: Joel Sussman & Dan Tawfik) The goal of this project is to develop high-throughput screening technologies for the rapid identification of biomolecules capable of intercepting chemical and biological war agents	1/6/2007 – 30/05/2009 Possible extension for another year $449,993 per annum	2.4 calendar
PENDING *(summarized for each individual)*		

Program Director/Principal Investigator (Last, first, middle): Tawfik,Dan S. (Project 3 , Lenz, David E.

GRANT NUMBER
5-U54-NS058183

CHECKLIST

1. PROGRAM INCOME ***(See instructions.)***
All applications must indicate whether program income is anticipated during the period(s) for which grant support is requested. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)
02		N/A
03		N/A
04		N/A

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***
In signing the application Face Page, the authorized organizational representative agrees to comply with the policies, assurances and/or certifications listed in the application instructions when applicable. Descriptions of individual assurances/certifications are provided in Part III of the PHS 398, and listed in Part I, 4.1 under Item 14. If unable to certify compliance, where applicable, provide an explanation and place it after the Progress Report (Form Page 5).

3. FACILITIES AND ADMINSTRATIVE (F&A) COSTS
Indicate the applicant organization's most recent F&A cost rate established with the appropriate DHHS Regional Office, or, in the case of for-profit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office.

F&A costs will ***not*** be paid on construction grants, grants to Federal organizations, grants to individuals, and conference grants. Follow any additional instructions provided for Research Career Awards, Institutional National Research Service Awards, Small Business Innovation Research/Small Business Technology Transfer Grants, foreign grants, and specialized grant applications.

☐ DHHS Agreement dated: ______________________ ☐ No Facilities and Administrative Costs Requested.

☐ No DHHS Agreement, but rate established with ______________________ Date __________

CALCULATION*

Entire proposed budget period: Amount of base $ 305,675 x Rate applied 8.00 % = F&A costs $ 24,054

Add to total direct costs from Form Page 2 and enter new total on Face Page, Item 8b.

*Check appropriate box(es):

☐ Salary and wages base ☐ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.):*